SOME TERATOGENIC EFFECTS OF TRYPAN BLUE AND ITS COMPONENTS ON DEVELOPING CHICKEN EMBRYOS

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GWENDOLYN P. CALLAWAY

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ABSTRACT

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Some Teratogenic Effects of Trypan Blue and Its Components on Chicken Embryos

Advisor: Dr. Roy Hunter, Jr.

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Gross observations showed that 0.1% concentration of trypan blue at a dosage of 0.5 cc was effective in producing growth retardation in 38% of 456 experimental embryos injected after 72 hr of incubation with whole trypan blue. Of the three fractions composing trypan blue, the blue fraction was found to be teratogenic, producing growth retardation and microphthalmia in 5.5% and 0.4% of 100 experimental embryos, respectively. The purple and red fractions compared with the saline injected embryos in exemplifying non-teratogenic activity.

Histochemical findings showed that the abdominal region exhibited more acid phosphatase activity in 4-day-old embryos injected with whole trypan blue and the blue fraction. Alkaline phosphatase activity was less in the abdominal region of 4-day-old embryos injected with whole trypan blue and the blue fraction.

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CHAPTER I

INTRODUCTION

Teratology is the branch of embryology which deal with malformations. It is the study of abnormal development. Experimental mammalian tertology began in the 1930's. However, the science of experimental teratology, practiced in lower classes of animals, was at least 100 years old. Successful experiments on avian and amphibian eggs were performed during the 19th and 20th centuries, but there were doubts as to whether the information obtained from these experiments could be applied to mammalian or human situations. Abnormalities which occurred in man or other mammals were thought to be hereditary because the embryos were so well protected by the maternal organism that they could not be modified by the teratogenic methods, as exemplified in lower classes (Wilson and Warkany, 1964).

According to these same authors, the science of nutrition furnished the early tools for this task. Vitamin A, riboflavin, and vitamin E were among the first experimental teratogenic tools used regularly for production of congenital malformations in mammals. Malnutrition and its effects on the fetus had been of interest to several investigators for a long time. Thus, Hale (1933) showed that a Registered Duroc-Jersey gilt, receiving a ration deficient in vitamin A,

farrowed 11 pigs, all of which were born without eyeballs. Of the ten pigs alive at birth, one lived four days, another 3 hours, while the others died within five minutes after birth. Therefore, they concluded that since both parents were normal eyed, the defect was not a hereditary one. This experimental demonstrated how a vitamin deficiency could have a profound effect on development.

Not only was there interest in the science of nutrition as providing tools for teratogenic experiments, but shortly after World War II there was a surge of interest as to the effects of X-ray as a teratogenic tool. Russell (1950) reported the effects of hard ionizing radiation as an injurious agent in mammalian development because of its tremendous penetration powers. From the data obtained, he concluded that the two main types of effects following irradiation were: (1) prenatal mortality, and (2) abnormality at birth.

In 1948, nitrogen mustard and trypan blue were shown to be positive teratogens. Haskin (1948) showed that the effects stimulated by nitrogen mustard were similar to those produced by X-ray and other penetrating radiations. There are three important aspects of their activity: (1) Mitotic activity is inhibited by action upon the interplase nucleus. (2) The most active proliferating cells may be specifically inhibited. (3) The action of nitrogen mustard is a rapidly completed one.

Gillman et al. (1948) introduced trypan blue as a teratogen. Young rats exposed to this dye exhibited such abnormalities as hydrocephalus, spina bifida and other congenital abnormalities. According to Wilson and Warkany (1964) trypan blue is still today a favorite teratogen used by many investigators; however, its mode of action is still unexplained.

Therefore, in view of this information, it is apparent that all abnormalities or malformations are not hereditary and passed from generation to generation. This proves that adverse environmental conditions resulting from vitamin deficiency, technological apparati, and industrial chemicals may modify offsprings if they are subjected to these teratogens. Thus, interest in the Science of Teratology has inspired the present investigator to look further into the effects of trypan blue, using chick embryos as test materials.

Trypan blue is the sodium salt of the blue disazo dye and has been used extensively as a vital stain. The dye is composed of a red, blue and a purple component. Each of these components has been found to differ in the amount of teratogenic activity. The literature is scant as it relates to the effects of the fractionated components of trypan blue on chick embryos. Likewise, so is the information on enzyme activity caused by the presence of the whole dye and its different fractions in the same animal. Thus, the purpose

of this investigation was to establish some of the gross malformations and histochemical effects of whole and fractionated trypan blue on developing chick embryos.

CHAPTER II

REVIEW OF LITERATURE

Trypan blue is the sodium salt of the blue disazo dye, prepared by coupling tetrazotized o-toludine with 2 molecular proportions of 8-amino-1napthol-3, 6-disulphonic acid. According to Beck and Lloyd (1963), this dye has been extensively used as a vital stain, particularly for the cells of the reticuloendothelial system and of the proximal convoluted tubules of the kidney.

Gillman et al. (1948) showed the teratogenic effects of trypan blue after injecting it into pregnant rats. Some of the abnormalities produced by this dye were hydrocephalus, spina bifida, eye defects, club foot, cleft palate and dislocation of fore- and hind-limbs. Similar abnormalities have also been shown in mice. Hamburgh (1952) observed that some of the abnormalities caused by trypan blue in mice are: abnormally broad heads, enlarged pericardia, retardation of the postumbilical body, and opened neural tubes. Waddington and Perry (1956) reported the teratogenesis of trypan blue in amphibian embryos. These investigators used Xenopus, axolotl, Triturus palmatus, and T. alpestris. The organisms were placed in different dilutions of trypan blue in 10% Holtfreter solution for varying lengths of time. Some of the abnormalities were edema, microcephaly, prevention of elongation

of the tail, failure gastrulation and mesodermalization of the notochord. Howe-er, inspite of the extensive study, little information reported has confirmed the underlying mechansim or site of action of the dye. Beaudoin and Wilson (1958) proposed 3 general types of action for the dye: It may (1) produce a change in the maternal metabolism which secondarily effects the embryo; (2) cause a blockage or alteraction of the placental transfer mechanism, or (3) have a direct action on the embryo. They showed that there is a direct action of trypan blue by using chick embryos as experimental animals.

Beck (1961) reported that of the two commercial preparations of trypan blue sold by Flatters and Garnett, and a batch sold by. G. T. Gurr, most of the activity was found in the Flatters and Garnett batch one. The second batch, Flatters and Garnett batch two, was highly active but not as much as batch one. However, the teratogenesis of the Gurr sample was not much different from the saline injected rats. Therefore, he concluded that commercial trypan blue is not a pure dye; that it is a mixture of substances, and one of a combination of more than one of the components was teratogenic.

Dijkstra and Gillman (1961) reported that there are differences in the biological activity of various commercial preparations of trypan blue. They also speculated that there are variations in the amount and kind of impurities present.

This was confirmed when a red fraction (violet) was found to be highly inactive and did not produce congenital malformations in rats. A Merck commercial preparation of trypan blue was separated on an alumina column and four fractions were obtained: red, blue, water soluble purple, and sodium hydroxide soluble purple. The red fraction was found inactive, and the blue fraction showed only slight activity. However, the purple fraction was highly active in inducing reticulosis in rats. Their results showed that so far as proliferation of the reticuloendothelial cells of the rat liver is concerned, the activity was associated with the purple fraction.

Barber and Geer (1964) used Dijkstra and Gillman's method of separating the dye on alumnia columns and obtained four fractions: blue, red, water-soluble purple and sodium hydroxide soluble purple. Whe injected into mice at 7, 8, and 9 days of gestation, only the blue fraction was teratogenic. Also, they found the dye to be time specific, in that the peak of teratogenic action was reached during the 7th, 8th and 9th days of gestation. After the 9th day of gestation, the activity ceased abruptly. The dyes used in these studies consisted of trypan blue, C. I. 447; an enriched red sample, B. C. 27775, and a low red sample, B. C. 27776, received from the National Aniline Division of Allied Chemical and Dye Corporation, New York.

Even though most of the information obtained about trypan blue has been through the use of mammalian systems, chick embryos have been used by several investigators. Kaplan and Grabowski (1967) injected fertile eggs of the Kimber strain of White Leghorn chickens into the yolk sac after 48 hours of incubation and found a high incidence of rumplessness at later stages of development. Their results indicated that hematomas played a role in the genesis of caudal defects. Most embryos opened after 3 days of dye treatment exhibited hematomas at the caudal end. However, after 5 days most of the embryos were found to be rumpless. Other effects were growth retardation, edema, clear blisters, bilateral or unilateral microphthalmia, hematomas in the brain or eyes, and others.

Hoffman and Ramm (1967) showed that there are physiological effects of trypan blue in chick embryos. They injected 10-day embryos with 0.1 ml of 1.5% trypan blue in sterile saline into the yolk sac. When the embryos were examined later, the treated ones exhibited a decreased survival rate, and a decrease in plasma glucose, chloride content, CO_2 content and blood pH. However, there was an elevation of plasma sodium or hematocrit. With saline injected embryos the survival rate decreased only slightly and there was a small but noticeable decrease in plasma chloride. Their results confirmed the evidence that the responses to trypan blue were similar

to those of hypoxia. Additionally, their data supported the view that trypan blue interfered with normal oxygen uptake.

Ferm and Beaudoin (1960) showed that when the yolk sac epithelium of the rat placenta is explanted to the chorio-allantoic membrane, this structure concentrates the azo dyes and bovine azo protein in much the same way and degree as in the intact animal. These results indicated that the absorptive capacity of rat yolk sac placenta is an inherent function of the cells themselves and is not dependent on its interposition between mother and fetus.

Later, a study by Beck (1964) suggested that the enzymes of the acid hydrolase group are concerned to some extent with the utilization of yolk by the developing chick. According to him, little is known of the distribution of these enzymes in the early chick blastoderm. A study was conducted of macroscopic and microscopic localization of acid phosphatase in chick explants at the 10 somite stage of development. Microscopic examination of frozen sections showed that acid phosphatase is present in the ectodermal cells forming the margin overgrowth of the blastoderm. The area vitellina was not as strongly positive as the blastoderm edge; however, much of the activity present was associated with yolk globules. The area vasculosa was strongly phosphatase positive, with much of the activity situated in the cytoplasm of the endodermal cells. The area pellucida showed little if any phosphatase activity and there was no uptake by trypan blue.

CHAPTER III

MATERIALS AND METHODS

Fertile eggs of White Leghorn chickens were obtained from a local hatchery. They were set out horizontally at room temperature for 24 hr prior to the beginning of incubation. All eggs were swa-bed with 70% ethanol and incubated at 37.8 C. They were allowed to incubate for 48, 72, and 96 hr. At these times, eggs were removed from the incubator, re-swabbed with 70% ethanol and a small opening was made near the blunt end with a sterile dissecting needle. A dosage of 1.) ml and 0.5 ml of a 0.1% solution of trypan blue, disolved in 0.85% sterile saline, was injected into the albumen of each egg via a 20 gauge needle attached to a 3 cc syringe. Controls were injected with 1.0 ml and 0.5 ml of 0.85% sterile saline. All eggs were swabbed around each opening, sealed with cellophane tape and returned to the incubator in a horizontal position. The eggs were turned at least twice a day prior to being opened. Some were opened 24 and 48 hr following injection. Before fixing, crown-rump length measurements were taken of control and experimental embryos with a metric ruler. No eggs were opened after seven days of incubation.

An Ames Cryostat was used to obtain frozen sections for histochemical analyses. The procedures were taken from the Sigma Company Technical Bulletin No. 385 and No. 85 for acid phosphatase and alkaline phosphatase, respectively.

A thin, concentrated line of a 0.1% solution of trypan blue was placed on Whatman chromatography paper. This paper was placed in a chromocabinet containing a solution of n-butanol-pyridine and water 10:50:40 (Beck and Lloyd, 1963). This solution separated the dye into 3 fractions: red, blue and purple. Each fraction was eluted from the paper with 30% aqueous pyridine. Fractions were then filtered, using Whatman no. 5 filter paper, to remove paper fibers. Each solution was filtered into a small vial, dried in a vacuum dessicator, and redissolved in sterile saline. Vials were weighed before and after drying to determine the quanity of the dye present.

Several eggs received injections of 1 ml and 0.5 ml of a 0.1% solution of each fraction except the purple fraction. All eggs injected with this fraction received 0.2 ml of a 0.1 solution. Histochemical procedures were the same as those for the whole dye trypan blue.

CHAPTER IV

EXPERIMENTAL RESULTS

The first of the data obtained from this research is concerned with the macroscopic observations of embryos exposed to trypan blue or its components. Information on histochemical analyses of control and experimental embryos will follow.

Macroscopic Observations

Controls

Observations were made on control embryos 48, 72, and 96 hr after incubation with 0.85% sterile saline. Out of a total of 375 eggs incubated at 37.8 C for 48 hr (Table 1), 275 survived and exhibited normal development; 25 were dead, and 75 showed no development at all. Crown-rump length measurements were made on embryos opened on the 3rd through the 7th day of incubation. They ranged from 0.8 cm to 2.7 cm on the initial and terminal days mentioned.

Whole Trypan Blue Injected Embryos

A total of 762 eggs was injected 48, 72, and 96 hr after incubation with 0.5 cc of 0.1% whole trypan blue dissolved in 0.85% sterile saline. Malformations were present as early as 24 hr after injection.

The 231 embryos injected 48 hr after incubation (Table 1) exhibited rumplessness, growth retardation from crown to rump, micropthalmia, and red hematomas. Examples of these conditions are shown in Figs. 1-5. The most frequently occuring malformation in embryos injected 48 hr after incubation with the whole dye was rumplessness.

Table I. Effects of whole trypan blue on chicken embryos injected after 48 hr of incubation.

	No. embryos injected	No. of embryos that did not develop or survive	Malformation (No. and %) 48 hr of incubation				
			Growth retardation	Microphthalmia	Rumpless ness	Hematomas	No visible malformation
Controls	375	100(27%)	0	0	0	0	275(74%)
Whole trypan blue	231	99(42%)	15(6.0%)	3(1.3%)	63(27%)	23(9.9%)	28(13.4%)
Total	606	199	15	3	63	23	313

Fig. 1. Growth retardation and rumplessness in chick embryos injected with whole trypan blue after 48 hr of incubation. These conditions are present in the experimental embryo (right). The control embryo (left) is longer than the experimental embryo.

Fig. 2. Growth retardation in embryos injected with whole trypan blue after 48 hr of incubation. The control embryo (right) is considerably longer than the experimental embryo (left).

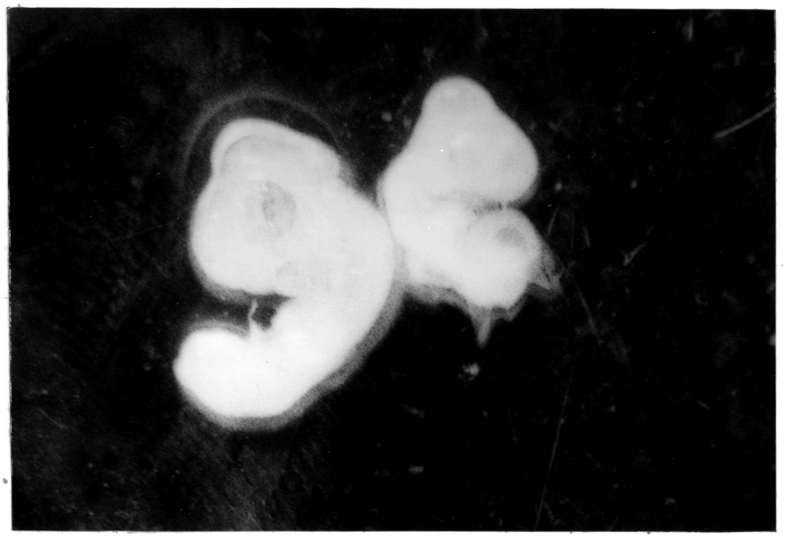
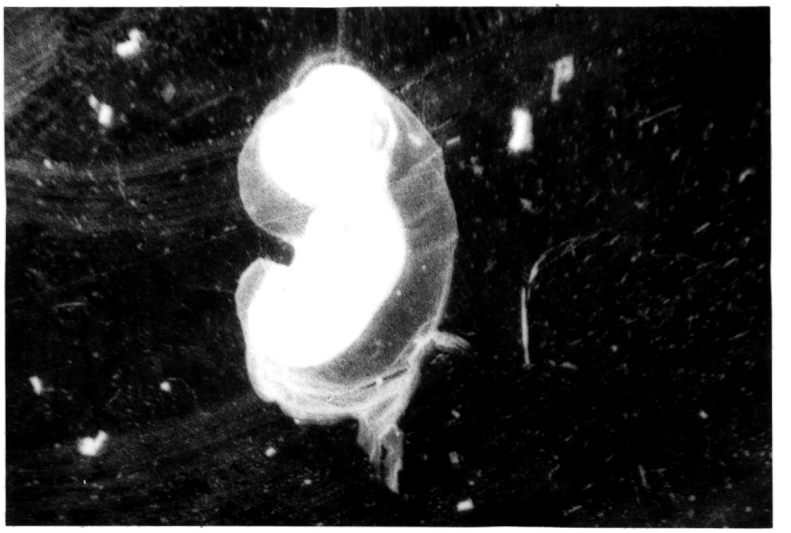


Fig. 3. Microphthalmia in embryos injected after 48 hr of incubation with whole trypan blue - front view.
This photograph also shows distortion in the telencephalic region of the brain.

Fig. 4. Microphthalmia and telencephalic distortion in 48 hr embryo injected with whole trypan blue - back view.



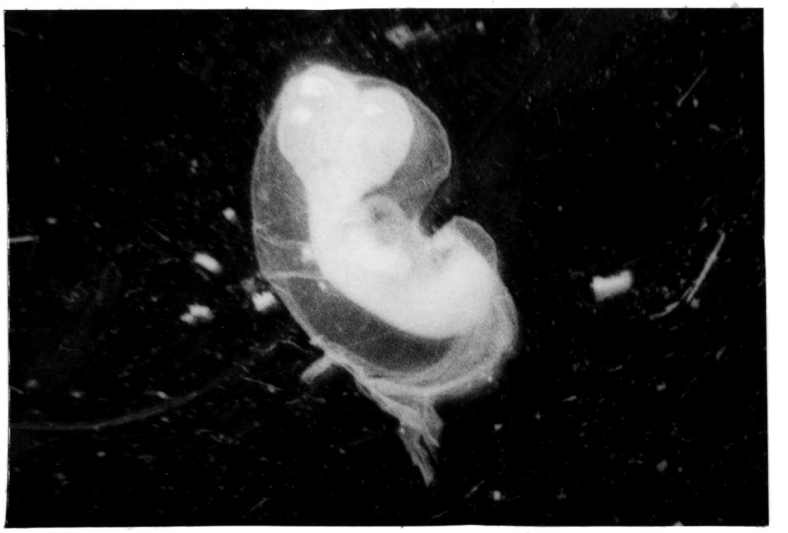
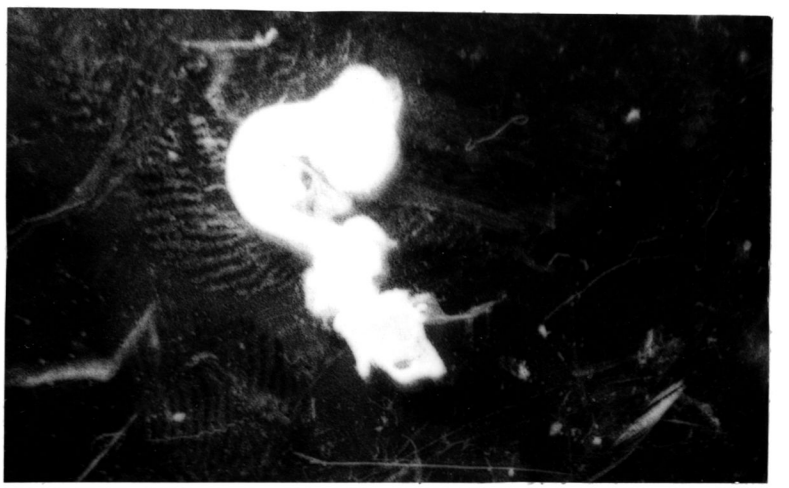


Fig. 5. Gross body distortion in 48 hr embryo injected with whole trypan blue. The blister at the caudal end is a red hematoma.



Of the 456 eggs injected 72 hr after incubation (Table 2), 172 exhibited growth retardation from the crown to rump regions; 5 had micropthalmia; 203 were dead or did not develop, and 76 survived without any malformation. Crown-rump length measurements of eggs opened 24 hr after injection and terminated on the 7th day of incubation ranged from 0.3 cm to 2.7 cm. Examples of these conditions are shown in Figs. 6-7. The 293 controls showed 52% normal development.

No malformations were found in embroys injected 96 hr after incubation at the same concentration and dosage. Of 75 injected eggs, 62 survived without any malformation and 13 died or did not develop.

Embryos Injected With Trypan Blue Fractions

The whole dye, trypan blue, separated off into three fractions when the ascending paper chromatography method used by Lloyd and Beck (1963) was employed. The dye is composed of blue, purple and red fractions (Fig. 8).

A total of 100 eggs was injected with this fraction (Table 2). Of these embryos, 23 exhibited growth retardation; 2 had microphthamia; 53 were dead or did not develop, and 20 showed no visible malformations (Figs. 9-10). As in the embryos injected with the whole dye, these embryos also received 0.5 cc of 0.1% blue fraction dissolved in 0.85% sterile saline. No eggs were injected with this fraction after 48 hr of incubation.

Table 2. Effects of trypan blue and its fractions on chicken embryos injected after 72 hr of incubation

	No. of embryos injected	No. of embryos that did not develop or survive	Malformations (No. and %) 72 hr after incubation		
			Growth retardation	Microphthalmia	No visible malformation
Controls	293	40(14%)	0	0	153
Whole trypan blue	456	203(45%)	172(38%)	5(1.2%)	76(17%)
Trypan blue fraction	100	53(53%)	25(5.5%)	2(0.4%)	20(20%)
Trypan red fraction	100	21(21%)	0	0	70(79%)
Trypan purple fraction	100	86(86%)	0	0	14(14%)
Totals	1,049	403	197	7	342

Fig. 6. 72 hr embryos injected with whole trypan blue; note growth retardation in the experimental embryo (left) as compared to the control embryo (right).

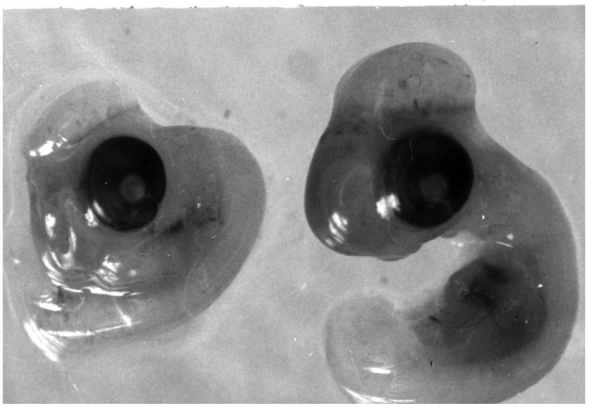


Fig. 7. Growth retardation in embryos injected with whole trypan blue 72 hr after incubation. This condition is shown in the experimental embryo (right).

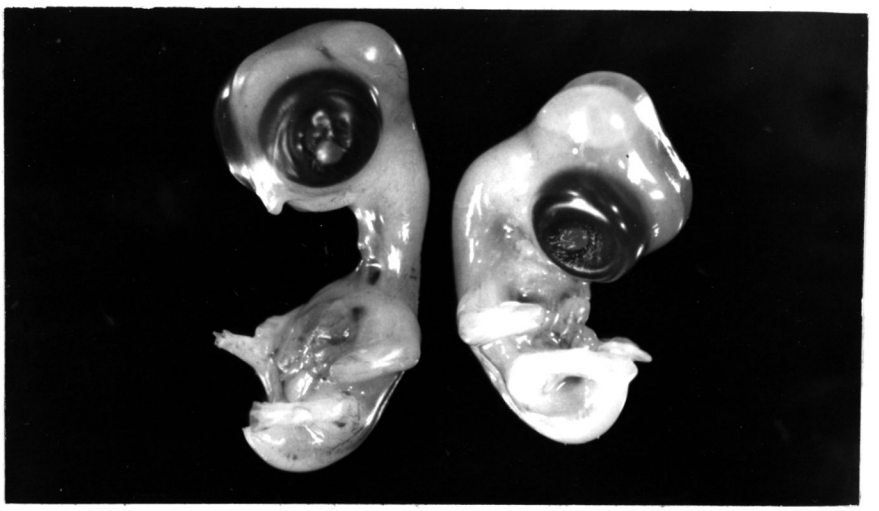


Fig. 8. Chromatographic separation of 0.1% Trypan Blue by ascending paper chromatography, yielding three fractions: blue, red or violet, and purple, respectively (from top to bottom).

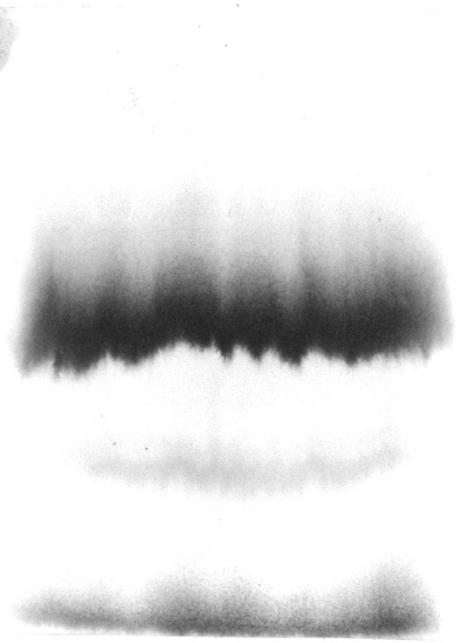
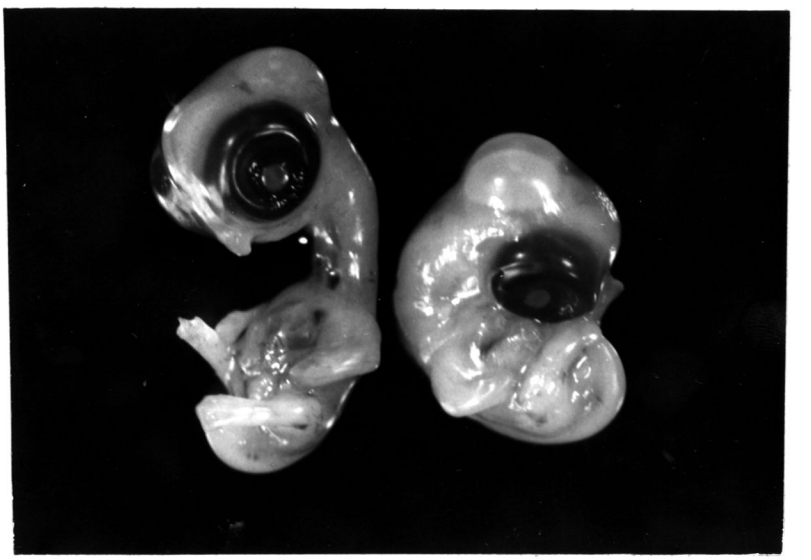


Fig. 9. Growth retardation in embryo injected with trypan blue fraction at 72 hr. The condition is exemplified in the experimental embryo (right).

Fig.



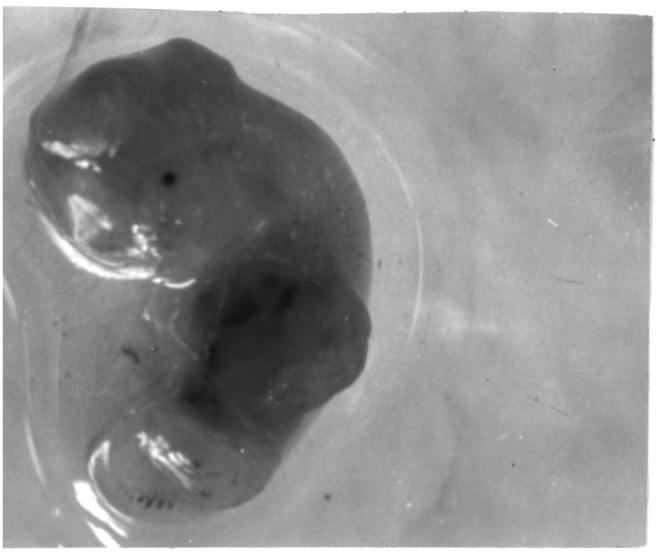


Fig. 11. Saline and purple fraction injected embryos. There are no considerable differences between the saline injected embryo (left) and the experimental embryo (right).

Fig.

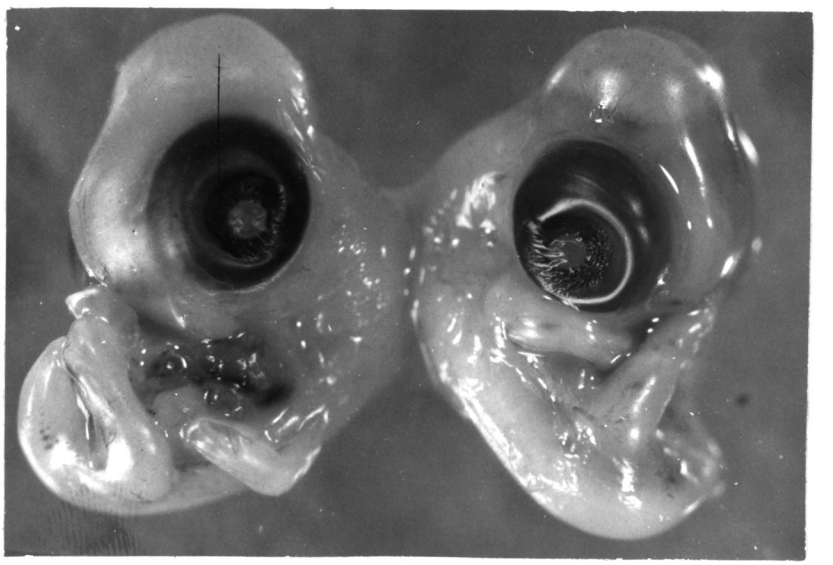


Fig. 12. Saline and red fraction injected embryos after 72 hr of incubation. There are no considerable differences between the saline injected embryo (right) and the experimental (left).

Table 2, 79 embryos exhibited normal development and only 21 died or did not develop. No significant differences existed between embryos injected with either the purple fraction (Fig. 11), and red fraction (Fig. 12).

Histochemical Analyses

Frozen sections were made of embryos on the 4th, 5th and 6th days of incubation for histochemical analyses. These sections were cut at 6μ and incubated for acid and alkaline phosphatase. The color intensity created by the azo-dye-coupling method was used as an index for the amount of enzyme activity exhibited by each enzyme present in the various sections. Color intensities were defined by plus signs as to the amount of activity. An intense activity is denoted by three plus signs, a moderate activity by one plus sign, and the intermediate activity by two plus signs.

Table 3 shows a comparison of the color intensities for alkaline phosphatase in saline, trypan blue and its components treated embryos on the fourth day of incubation. As the table shows, more activity is present in the saline, red fractions and purple fraction injected embryos in the whole dye and the blue fraction. However, as shown in Table 4, more acid phosphatase activity occurred in the abdominal region than in the tail region of whole trypan blue and trypan blue fraction injected embryos. Not much activity occurred in the tail regions of these embryos. For the 5-and 6-day-old embryos, the results remained the same for both alkaline and acid phosphatase.

Table 3. Alkaline Phosphatase Activity in 4-day-old embryos.

Saline and dye injected embryos	*Color intensities of embryo sections	
	abdominal regions	tail regions
Saline	++	++
Whole trypan blue	+	+
Trypan blue fraction	+	+
Trypan red fraction	++	++
Trypan purple fraction	++	++

*Two plus signs denote intermediate acitivty.

One plus sign denotes moderate activity.

Table 4. Acid Phosphatase Activity in 4-day-old embryos.

Saline and dye injected embryos	*Color intensities of embryo sections	
	abdominal region	tail region
Saline	++	++
Whole trypan blue	+++	+
Trypan blue fraction	+++	+
Trypan red fraction	++	++
Trypan purple fraction	++	++

*Three plus signs denote intense activity.
Two plus signs denote intermediate activity.
One plus sign denotes moderate activity.

CHAPTER V

DISCUSSION AND CONCLUSION

The gross observations have revealed that trypan blue produces different malformations at varying stages of development. In two-day-old embryos, rumplessness has been found to be the most frequently occurring malformation, with hematomas at the caudal end prior to rump formation. According to Kaplan and Grabowski (1967), hematomas play a role in the genesis of caudal defects. However, the results here have shown that growth retardation was the most frequently occuring malformation in embryos injected after 72 hr of incubation. The formation of the rump has already taken place at this time. At both stages of development, growth retardation, however, appeared more frequently after 72 hr than after 48 hr of incubation. Other malformations caused by this dye were microphthalmia and gross body distortions. The non-survival rate was greater in experimental embryos than in embryos injected with saline.

Of the three fractions composing trypan blue, the blue fraction was found to produce a greater percentage of malformations, with growth retardation from crown to rump being the most frequently occurring one. Beck and Lloyd (1963) showed that in rats, the most active constituent of commercial trypan blue was the blue fraction. Although growth retardation

was not present in this experimental animal, microphthalmia was found to be one of the external malformations following treatment with the blue fraction. Deformed tails and tail-lessness were also present. These investigators, too, did not find the other impurities to be active teratogens. However, Dijkstra and Gillman (1961) found their purple fraction to be the active component of commercial trypan blue in rats. Later, Barber and Geer (1964) injected the fractions into mice and found only the blue fraction to be teratogenic. Another important factor was the fact that the dye was found to be time specific, the peak of teratogenic activity was reached during the 7th, 8th, and 9th days of gestation. Even though the purple fraction was not found to be teratogenic in the present investigation, it was more lethal at the same dosage as that of trypan blue and the two other fractions. Therefore, the purple fraction was found to be sublethal at a lower dosage. This may be an indication of its teratogenicity but not in the chick embryo.

Histochemical analyses for the enzymes, acid and alkaline phosphatase, were performed to assess some facets of trypan blue. No conclusions can be drawn as to the significance of these studies as they relate to the gross malformation caused by trypan blue and its fractions. However, there are speculations that there is lysosomal enzyme inhibition by trypan blue. The action of trypan blue inhibition of

embryotrophic nutrition was suggested and exemplified on pregnant rats. There is an indication that the dye is an inhibitor of a selection of hydrolytic enzymes present in lysosmal fractions from homogenates of rat visceral yolk sac (Beck, Lloyd, and Griffith, 1967). If so, then, the data (see Table 4) may be of significance and should receive futher study.

Therefore, it can be concluded that different commercial preparations of trypan blue vary in their amount of teratogenic activity. Different stages of development, too, play a role in determining the teratogenic activity of trypan blue and its components.

CHAPTER VI

SUMMARY

1. This study has confirmed the fact that a teratogenic industrial chemical, in this case trypan blue, may modify offsprings at varying stages of development. After 48 hr of incubation, rumplessness occurred more frequently in chicken embryos. Of the 231 eggs injected with whole trypan blue, 27% of the embryos exhibited rumplessness. However, after 72 hr, growth retardation from crown to rump was the most frequently occuring malformation. Of the 456 eggs injected with whole trypan blue, 38% exhibited growth retardation from the crown to rump regions.

2. It was found that the commercial preparation of trypan blue used in this investigation was composed of three fractions: a blue, purple, and a red (violet) fraction.

3. Of the three fractions composing trypan blue, the blue component was more active in producing external malformations in 72 hr chicken embryos. Of a total of 100 eggs injected with this fraction, 5.5% of the embryos exhibited growth retardation. The red and purple impurities were not found to be teratogenic. However,

the purple fraction was more lethal at the same dosage as that of the whole dye and the fractions. A tabulation has been made of the kinds and frequencies of abnormalities produced by trypan blue and its components.

Histochemical analyses have shown that alkaline and acid phosphatase activity present in the abdominal and tail regions of 4-day-old embryos. It was found that there was more acid phosphatase activity in the abdominal region of 4-day-old embryos, injected with whole trypan blue and the blue fraction. On the otherhand, alkaline phosphatase was lesser in the abdominal and tail regions of embryos injected with whole trypan blue and its fraction.

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